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The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events

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SecA is the ATP-dependent force generator in the *Escherichia coli* precursor protein translocation cascade, and is bound at the membrane surface to the integral membrane domain of the preprotein translocase. Preproteins are thought to be translocated in a stepwise manner by nucleotide-dependent cycles of SecA membrane insertion and de-insertion, or as large polypeptide segments by the protonmotive force (Δp) in the absence of SecA. To determine the step size of a complete ATP- and SecA-dependent catalytic cycle, translocation intermediates of the preprotein proOmpA were generated at limiting SecA translocation ATPase activity. Distinct intermediates were formed, spaced by intervals of ~5 kDa. Inhibition of the SecA ATPase by azide trapped SecA in a membrane-inserted state and shifted the step size to 2–2.5 kDa. The latter corresponds to the translocation elicited by binding of non-hydrolysable ATP analogues to SecA, or by the re-binding of partially translocated polypeptide chains by SecA. Therefore, a complete catalytic cycle of the preprotein translocase permits the stepwise translocation of 5 kDa polypeptide segments by two consecutive events, i.e. ~2.5 kDa upon binding of the polypeptide by SecA, and another 2.5 kDa upon binding of ATP to SecA.

Keywords: energetics/protonmotive force/SecA/secretion

Introduction

Preprotein translocation across the cytoplasmic membrane of *Escherichia coli* is mediated by a multisubunit enzyme termed translocase (Wickner *et al.*, 1991; Driessen, 1994). It consists of the cognate heterotrimeric integral membrane domain with SecY, SecE and SecG as subunits, the peripheral ATPase SecA, and SecD and SecF as associated integral membrane proteins (Duong and Wickner, 1997a). The translocation of preproteins requires two forms of energy, ATP and the protonmotive force (Δp) (Geller *et al.*, 1986; Geller and Green, 1989; Yamada *et al.*, 1989a). Although both forms of energy are essential *in vivo*, *in vitro* translocation can be driven by ATP hydrolysis alone provided that SecA is present in excess (Yamada *et al.*, 1989b). SecA is a large homodimeric protein (M_r 102 kDa) (Schmidt *et al.*, 1988) with two distinct folding domains, i.e. an N- and C-terminal domain (Den Blaauwen *et al.*, 1996). SecA has two essential nucleotide-binding sites (NBSs; Mitchell and Oliver, 1993) that function in a cooperative manner. The high-affinity NBS-I ($K_{d,ADP}$ ~50 nM) resides in the N-terminal domain, whereas the low-affinity NBS-II ($K_{d,ADP}$ ~300 μM) is localized in the C-terminal domain. SecA is the only ATPase involved in translocation, and its activity is stimulated by high-affinity interactions with preproteins and the SecYEG complex (Lill *et al.*, 1990). SecA interacts in a nucleotide-dependent manner with the membrane surface (Breukink *et al.*, 1992). The SecYEG-bound SecA is thought to insert into the membrane with a 30 kDa C-terminal domain (Price *et al.*, 1996; van der Does *et al.*, 1996) upon binding of ATP at both of its NBSs (Economou and Wickner, 1994; Economou *et al.*, 1995). This process also allows the limited translocation of a loop of the signal sequence and N-terminal mature region of the preprotein to the extent that the signal sequence can be processed by leader peptidase (Schiebel *et al.*, 1991). After insertion, release of the bound preprotein (Schiebel *et al.*, 1991) and de-insertion of the SecA domain from the membrane requires at least the hydrolysis of ATP at the high-affinity NBS-I (Van der Wolk *et al.*, 1993; Economou *et al.*, 1995), while hydrolysis of a second ATP molecule at NBS-II is needed to release SecA from the membrane (Economou *et al.*, 1995). Re-association of SecA with the partially translocated preprotein permits a new cycle of ATP-dependent membrane insertion and de-insertion of SecA which drives the limited translocation of another preprotein domain. The reaction can be completed by consecutive cycles of ATP-driven translocation (Schiebel *et al.*, 1991).

The mechanism by which the Δp accelerates the translocation reaction is only poorly understood. The Δp can act as the sole driving force for the completion of translocation when a partially translocated preprotein is not associated with SecA (Driessen and Wickner, 1991; Schiebel *et al.*, 1991; Driessen, 1992; Van der Wolk *et al.*, 1993; Duong and Wickner, 1997b). Other studies indicate that the Δp acts on the SecA translocation ATPase by promoting the release of ADP from SecA (Shiozuka *et al.*, 1990). The Δp has also been suggested to modulate the width of the translocation channel as, in its presence (plus ATP), short segments of proOmpA with a stable tertiary fold stabilized by a disulfide bridge can be translocated (Tani *et al.*, 1990). Strains carrying a signal sequence suppressor mutation in SecY (*prlA*) translocate such stabilized structures even without a Δp (Nouwen *et al.*, 1996b). With some precursor proteins, the Δp is required for the initiation of translocation (Nouwen *et al.*, 1996a). Mutations in the signal sequence or early mature region of the preprotein can either relieve or elevate the Δp requirement (Lu *et al.*, 1991; Geller *et al.*, 1993; Nouwen *et al.*, 1996a).

Based on the use of non-hydrolyzable nucleotide ana-

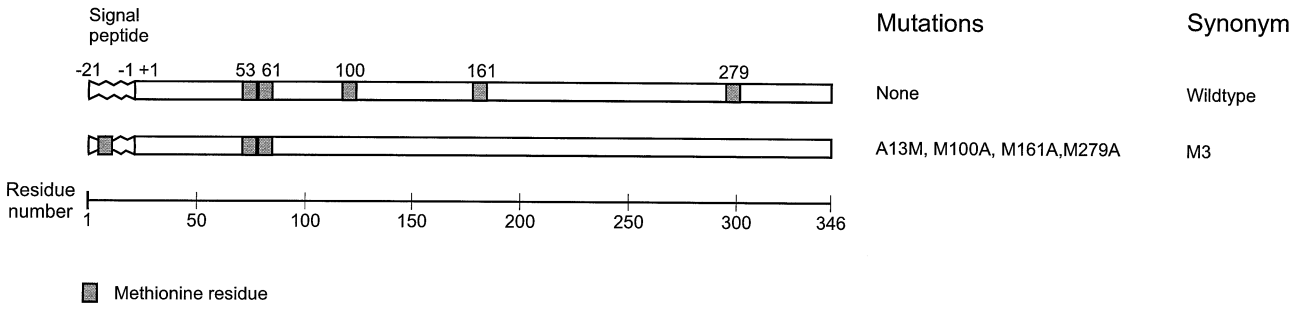

Fig. 1. Schematic representation of the proOmpA derivative. Amino acid residues of the signal peptide (–21 to –1) and mature (+1 to 325) regions are indicated with the positions of methionine residues (shaded squares). The name of the mutant proOmpA is indicated.

logues (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991), and the formation of translocation intermediates that have been arrested by the introduction of a stable tertiary structure in the molecule (Uchida *et al.*, 1995), it has been suggested that translocase mediates preprotein translocation with a step size (quantum) of ~2–2.5 kDa. Both methods, however, rely on the imposition of a synthetic translocation arrest. Here we report on the kinetic step size of an ongoing translocation reaction. A complete catalytic cycle of the SecA ATPase, which involves both binding of the preprotein and binding and hydrolysis of ATP, promotes the translocation of a polypeptide domain that is twice the size of a quantum.

Results

Formation of early intermediates in proOmpA translocation

Translocation intermediates allow a systematic analysis of the energetics of intermediate steps in protein translocation. ProOmpA, the precursor of the outer membrane protein OmpA, is a 336 amino acid polypeptide with an M_r of 38 kDa. Previously, late intermediates of proOmpA translocation have been described that were translocated for ~16, 26 and 29 kDa of the molecular mass (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991). These intermediates expose their C-terminus to the outer surface when translocated into inverted inner membrane vesicles (IMVs) of *E.coli*, and transiently accumulate under conditions where translocation is slow (i.e. low ATP concentration and absence of the Δp). To facilitate the identification of early intermediates, a mutant proOmpA was constructed that contains methionine residues in the N-terminal region only. The three C-terminal Met residues at positions 121, 182 and 300 (Figure 1) were replaced by alanine residues, and an extra Met was introduced in the signal sequence by replacing the Ala at position 13. This mutant proOmpA was termed proOmpAM3 (Figure 1). It translocates as efficiently into *E.coli* IMVs as does the wild-type protein (data not shown), and was used throughout the subsequent experimental work.

In the absence of a Δp, and at a low ATP concentration, translocation intermediates can be readily detected (Schiebel *et al.*, 1991). [^{35}S]proOmpA, SecA, SecB and urea-extracted IMVs of *E.coli* D10 were pre-incubated at 37°C to allow binding of proOmpA to the translocase (Hartl *et al.*, 1990). Next, 2 μM ATP were added to initiate slow translocation. In the presence of an excess of creatine kinase and phosphocreatine, the steady-state

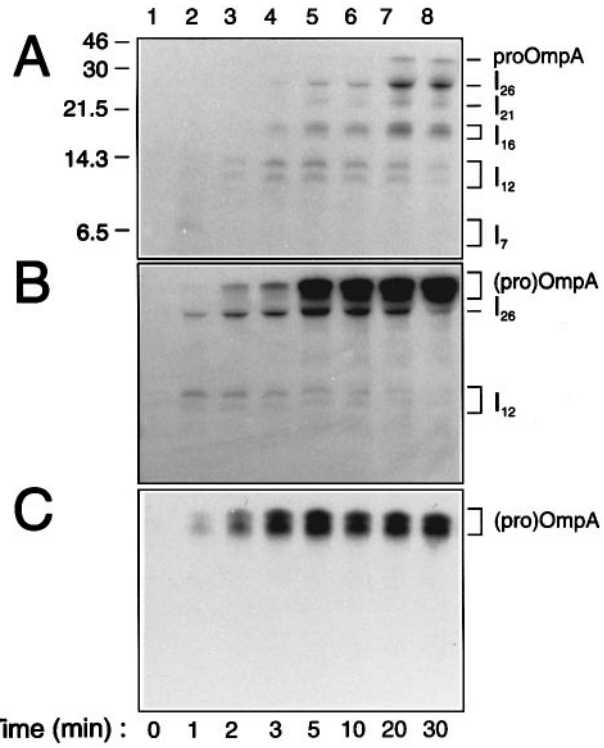

Fig. 2. Early intermediates in proOmpA translocation are formed at low ATP concentrations in the absence of a Δp. The translocation of [^{35}S]proOmpA into urea-treated IMVs derived from *E.coli* strain D10 was followed over time in the presence of 2 μM (**A**) or 2 mM ATP (**B**), and into native D10 IMVs in the presence of 2 mM ATP (**C**). Translocation reactions were performed as described in Materials and methods. At the indicated time points, samples were taken and proteinase K-protected fragments were analysed by Tricine gels and autoradiography.

ADP concentration was found to be <0.1 μM. The calculated nucleotide occupancy of the SecA NBS-I and -II under these conditions is 92 and 0.5%, respectively (Den Blaauwen *et al.*, 1996). At various times, samples of the reaction were taken and assayed for translocation intermediates by incubation on ice with proteinase K followed by SDS–PAGE using Tricine gels which are optimized for the separation of polypeptides with a molecular mass in the range of 3–30 kDa (Schägger and Von Jagow, 1987). After autoradiography, the successive appearance of proteinase K-protected proOmpA domains of increasing size was evident (Figure 2A). The species with M_rs of 16 (doublet of 15 and 17 kDa) and 26 kDa correspond to the intermediates I_{16} and I_{26}, respectively,

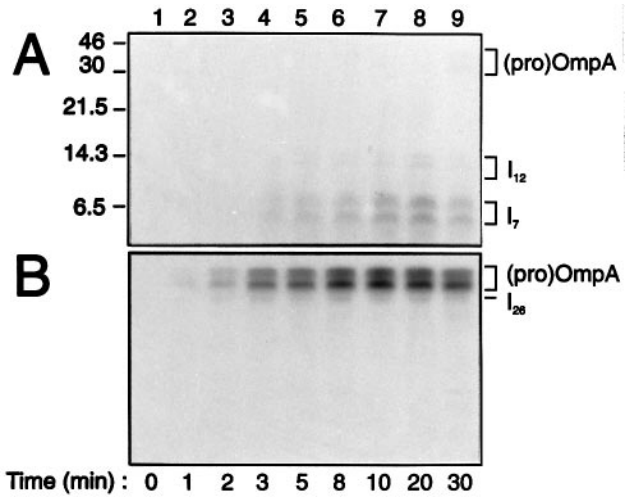

Fig. 3. Dissipation of the Δp slows down translocation at an early stage. Translocation reactions of [^{35}S]proOmpA in the presence of 2 mM ATP into native D10 IMVs were performed in the presence (**A**) or absence (**B**) of 10 μM CCCP. At the indicated time points, samples were taken and proteinase K-resistant fragments were analysed on a 16% Tricine gel, followed by autoradiography as described in Materials and methods.

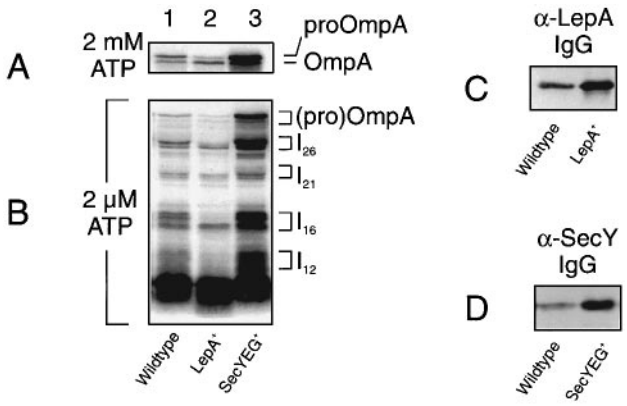

Fig. 4. Intermediates accumulate at the translocation sites as doublets. Translocation reactions of [^{35}S]proOmpA were performed for 10 min in the presence of 2 mM (**A**) or 2 μM ATP (**B**) using urea-treated IMVs derived from *E.coli* strain D10 cells (Wildtype) harbouring plasmid pTD101 (LepA$^+$) or plasmid pET324 (SecYEG$^+$). Reactions were analysed by 12% SDS–PAGE followed by autoradiography. Membrane vesicles were subjected to immunoblot analysis for the amounts of LepA (**C**) and SecY (**D**) using polyclonal antibodies raised against the purified proteins.

that have been characterized previously in great detail (Schiebel *et al.*, 1991). They typically appear as doublets in a narrow molecular mass range. Due to the limited resolution of the Tricine gel system, I_{26} is resolved only as a single species. At the early time points, protected proOmpA domains of 5, 7, 10 and 12 kDa are seen, whereas at later time points, a 21 kDa protected fragment is formed (Figure 2A). The rate of proOmpA translocation increases with the ATP concentration, and at 2 mM ATP (NBS-I and -II occupancy of 100 and 85%, respectively), mainly fully translocated (pro)OmpA is observed, with only small amounts of the early protected species of 10 and 12 kDa and I_{26}. None of the other protected species are observed, indicating that they are true kinetic intermediates (Figure 2B). These data suggest that in analogy with the late stage of translocation, early stages also proceed through an ordered series of intermediates, tentatively termed I_7 (doublet of 5 and 7 kDa) and I_{12} (doublet of 10 and 12 kDa). In addition to the previously characterized I_{16} and I_{26}, another, less abundant intermediate can be detected, termed I_{21}. In the presence of 2 mM ATP and a Δp, using native D10 IMVs, translocation is extremely rapid without the apparent appearance of intermediates (Figure 2C).

Dissipation of the Δp impedes translocation at an early stage

The ability to dissipate the Δp selectively with uncouplers was used to determine the role of Δp in the kinetics of the early stages of translocation. Translocation reactions were performed with native *E.coli* D10 IMVs bearing endogenous SecA. These IMVs generate a Δp in the presence of ATP through the activity of the H^+-translocating F_1F_0-ATPase, and exhibit extremely rapid proOmpA translocation without detectable accumulation of early intermediates (Figure 3B). When the Δp is dissipated by the addition of the uncoupler CCCP (Figure 3A) or a combination of the ionophores nigericin and valinomycin (data not shown), translocation is slowed down severely, and protected fragments of 5 and 7 kDa, and at later stages of 10 and 12 kDa, appear as major intermediate species. Unlike the urea-extracted *E.coli* D10 IMVs, translocation of proOmpA into the native IMVs in the presence of CCCP or valinomycin/nigericin could not be restored by the addition of an excess of purified SecA. Inactivation of the H^+-translocating F_1F_0-ATPase by DCCD also causes retardation of the rate of translocation, yielding the same early intermediates (data not shown). Since I_7 is the major species accumulating in the absence of a Δp, it appears that the initiation of translocation is only marginally Δp dependent while the following stages are strongly promoted by the Δp. This is consistent with the notion that the the Δp is not obligatorily required for initiation of proOmpA translocation (Geller and Green, 1989; Schiebel *et al.*, 1991).

Intermediates accumulate as doublets at the translocation sites

Translocation intermediates never appear as a unique species, but typically as a doublet. These doublets either represent the processed and non-processed form of a unique intermediate, or correspond to distinct intermediates that have been translocated to different extents. To discriminate between these two possibilities, IMVs were isolated from *E.coli* D10 harbouring plasmid pTD101 (Date and Wickner, 1981) that allows for a 3-fold overproduction of leader peptidase, LepA (Figure 4C). In urea-extracted LepA$^+$ IMVs, processing of fully translocated proOmpA to OmpA is greatly enhanced, but still not complete (Figure 4A, compare lanes 1 and 2). The intermediates formed during translocation in the presence of a low amount (2 μM) of ATP are similar in wild-type and LepA$^+$ vesicles, with the difference that the intensity of the upper band in the doublet is diminished in LepA$^+$ vesicles, most strikingly in the case of I_{16}. These data indicate that the formation of doublets is most likely due to incomplete processing by leader peptidase.

To assay the involvement of the integral membrane

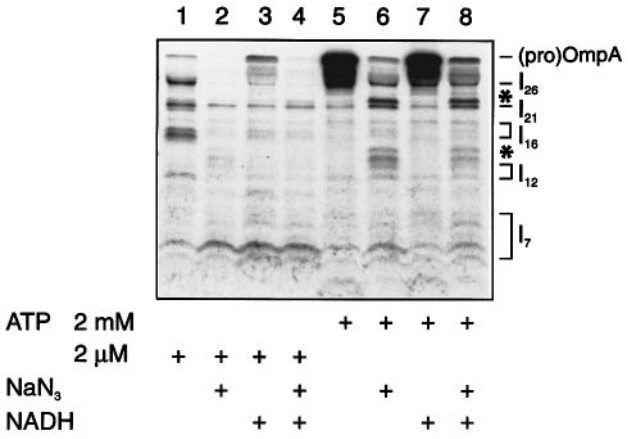

Fig. 5. Arrest of translocation by sodium azide results in the accumulation of novel translocation intermediates. Translocation of [^{35}S]proOmpA into IMVs derived from *E.coli* strain KM9 was performed for 10 min in the absence or presence of 20 mM azide (NaN_3) and/or 10 mM NADH to impose a Δp as indicated. Reactions were analysed on a 16% Tricine gel and the amount of (pro-)OmpA translocated was quantified by densitometric scanning of the films.

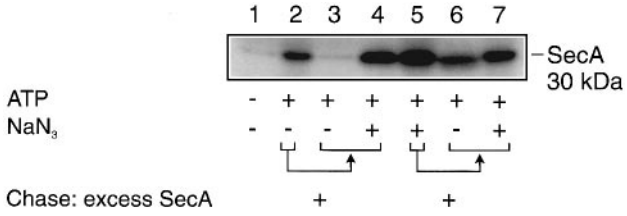

Fig. 6. Sodium azide prevents the de-insertion of SecA from the membrane. Insertion of SecA into the membrane was assayed by protease protection of the membrane-inserted 30 kDa fragment of [^{125}I]SecA (Economou and Wickner, 1994). [^{125}I]SecA (2.5 μg/ml) was incubated under translocation conditions in the presence of proOmpA (20 μg/ml) and YEG^+ IMVs (300 μg/ml), in the absence (lane 1) or presence (lanes 2–7) of 2 mM ATP and in the absence (lane 2) or presence (lane 5) of 20 mM NaN_3. After 10 min, aliquots were taken and incubated for an additional 10 min with an excess of SecA (50 μg/ml) which was pre-incubated without (lanes 3 and 6) or with (lanes 4 and 7) 50 mM NaN_3. Samples were incubated for 20 min with proteinase K (1 mg/ml) and analysed by SDS–PAGE (12%) and autoradiography.

domain of the translocase, translocation reactions were performed with urea-treated IMVs derived from *E.coli* D10 harbouring plasmid pET324, which allows for the high level overproduction of the SecYEG complex (van der Does *et al.*, 1996; Figure 4D). The rate of proOmpA translocation was enhanced in $SecYEG^+$ IMVs (Figure 4A, compare lanes 1 and 3; Douville *et al.*, 1995; Van der Does *et al.*, 1996), but the major intermediates, i.e. I_{12}, I_{16} and I_{21}, were formed with a much higher yield as compared with IMVs of the parental strain (Figure 4B, compare lanes 1 and 3). These data support the notion that intermediates accumulate at the translocation sites.

Azide traps SecA in a membrane-inserted state and induces the accumulation of novel intermediates

Sodium azide (NaN_3) is a potent inhibitor of protein translocation, although *in vitro* the inhibition is not complete (Oliver *et al.*, 1990). NaN_3 selectively blocks the preprotein-stimulated ATPase activity of SecA (translocation ATPase), and thus provides a convenient method to reduce the activity of SecA. Translocation reactions were performed with IMVs derived from strain KM9 that lacks the entire *unc* operon. These vesicles do not generate a Δp in the presence of ATP but can be energized with an oxidizable substrate such as NADH. In the presence of excess SecA and high ATP (2 mM), translocation of proOmpA into *E.coli* KM9 IMVs appeared to be less dependent on the presence of a Δp (i.e. addition of NADH) as compared with *E.coli* D10 IMVs. Under these conditions, only a few intermediates are detectable after 10 min of translocation (Figure 5, lanes 5 and 7). Addition of 20 mM NaN_3 severely retards but does not completely block translocation (lanes 6 and 8), giving rise to intermediates. Measurements of the magnitude of the transmembrane electrical potential (ΔΨ), inside positive, and pH gradient (ΔpH), inside acid, as measured with the fluorescent dyes oxonol VI and ACMA, respectively (not shown), show that the NaN_3 does not interfere with the Δp generation. Remarkably, when NaN_3 is present, the pattern of intermediates deviates from that typically observed. In addition to the previously described I_{12}, I_{16}, I_{21} and I_{26}, two extra abundant intermediates are observed, with estimated sizes of 13–15 and 23 kDa (indicated by asterisks, lanes 6 and 8). NaN_3 blocks proOmpA translocation completely at a low ATP concentration, i.e. 2 μM. In the absence of a Δp, the typical intermediates are formed after 10 min of translocation (Figure 5, lane 1), while the presence of a Δp dramatically stimulates translocation (lane 3). Under both conditions, NaN_3 prevents the formation of full-length proOmpA (lane 2 and 4), and, although much weaker, new intermediates were formed. These results indicate that NaN_3 interferes with the cycle of ATP- and Δp-driven translocation, possibly by trapping the translocase in a transition stage yielding novel intermediates.

To determine how NaN_3 affects the catalytic cycle of SecA membrane insertion and de-insertion, its effect on the formation of the protease-resistant and membrane-protected 30 kDa SecA fragment was investigated under translocating conditions, i.e. with urea-treated $SecYEG^+$ IMVs, proOmpA, 2 mM ATP and ^{125}I-labelled SecA (2.5 μg/ml) (Economou and Wickner, 1994). NaN_3 does not inhibit, but rather stimulates the formation of the 30 kDa SecA fragment (Figure 6, compare lanes 2 and 5), suggesting that NaN_3 stabilizes the membrane-inserted state of SecA. SecA membrane de-insertion can be measured in a chase experiment in which a 20-fold excess of non-labelled SecA is added to membranes bearing inserted [^{125}I]SecA. Under translocation conditions, the inserted [^{125}I]SecA is displaced rapidly by non-labelled SecA, which is evident from the disappearance of the 30 kDa SecA fragment after protease digestion (Figure 6, lane 3). NaN_3 efficiently prevents this chase (lanes 4 and 6), and this effect is even more pronounced when excess NaN_3-treated SecA is added (lane 7). These data suggest that NaN_3 inhibits preprotein translocation by trapping the SecA in a membrane-inserted state.

Discussion

Preprotein translocation across the inner membrane of *E.coli* requires two energy sources, ATP and the Δp

(Wickner *et al.*, 1991; Driessen, 1992). ATP is essential for translocation, while the Δp stimulates the rate of translocation. Previous *in vitro* studies on late translocation intermediates have shown that the ATP-driven translocation of proOmpA is a stepwise process (Schiebel *et al.*, 1991; Uchida *et al.*, 1995). We now show that the stepwise character of SecA-driven translocation is also manifested during early steps of translocation, and have determined the functional step size of the catalytic cycle of SecA-mediated and ATP-driven translocation.

The SecYEG-bound form of SecA performs a key role in the preprotein translocation process. It binds the preprotein by direct recognition of the signal sequence and mature domain (Cunningham and Wickner, 1989; Lill *et al.*, 1990), and uses the energy of ATP binding and hydrolysis to drive the preprotein translocation reaction (Schiebel *et al.*, 1991). SecA has two essential ATP-binding sites, i.e. NBS-I and NBS-II, that bind nucleotides with high and low affinity, respectively (Mitchell and Oliver, 1993). When both NBSs are saturated with ATP, preprotein translocation is fast and intermediates in translocation accumulate only at a late stage. At NBS-II subsaturating concentrations of ATP, the catalytic cycle of SecA is dramatically retarded and the translocation of the preprotein proOmpA is slow. Under those conditions, a series of discrete intermediates accumulate. Based on the SDS–PAGE analysis and comparison with molecular mass standards, translocation intermediates seem to appear in intervals of ~5 kDa. Depending on the translocated polypeptide mass, these intermediates are tentatively named: I_7, I_{12}, I_{16}, I_{21} and I_{26}. The late intermediates I_{16} and I_{26} have been described before (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991), and in everted IMVs expose their non-translocated C-terminus to protease added from the outside. Our specifically [^{35}S]methionine-labelled proOmpA molecule, in combination with a high resolution Tricine gel system, allowed the detection of the early intermediates I_7 and I_{12} that after protease digestion occur as short polypeptides. In addition, a weak late intermediate I_{21} could be detected. The systematic occurrence of intermediates in intervals of ~5 kDa suggests that the functional step size of translocation may be larger than the 2–2.5 kDa translocation elicited by the binding of a non-hydrolysable ATP analogue to SecA (Schiebel *et al.*, 1991). However, the latter step size is determined under conditions where the catalytic cycle of the translocase is blocked as hydrolysis of the SecA-bound ATP is not possible. Hydrolysis of ATP is needed to release the preprotein from SecA, whereupon SecA can re-enter the translocation reaction by binding the non-translocated portion of the preprotein. Schiebel *et al.* (1991) have shown that this re-binding reaction already permits the translocation of a 2–2.5 kDa polypeptide segment. Subsequent binding of a non-hydrolysable ATP analogue to SecA effects the translocation of another 2–2.5 kDa of the polypeptide mass (Schiebel *et al.*, 1991). Uchida *et al.* (1995) have shown that the translocation of proOmpA can be arrested at intervals of ~2.5 kDa by the introduction of disulfide bridges in the molecule. Upon oxidation, a stable tertiary structure is formed in the molecule that cannot be translocated. This observation has led to the suggestion that the catalytic cycle of the translocase allows the translocation of ~2.5 kDa, superficially termed a quantum. Since this estimate is based on a synthetically imposed translocation arrest, it cannot be ascertained that the estimated step size indeed reflects an entire catalytic cycle of the translocase. Rather, it agrees with the step size defined for the partial reactions as described by Schiebel *et al.* (1991).

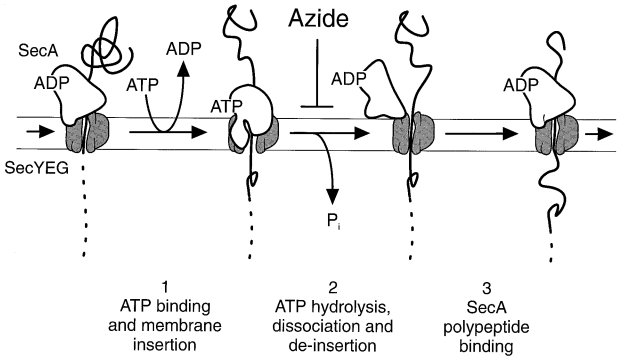

Fig. 7. Model for the stepwise mode of action of precursor protein translocation. See Discussion for details.

Based on the above considerations, we propose that a complete catalytic cycle of SecA, i.e. binding and hydrolysis of ATP, involves two distinct translocation steps that together permit ~5 kDa of translocation progress (Figure 7). Upon ATP binding, the SecA bound to the partially translocated polypeptide chain will translocate ~2.5 kDa of the precursor protein concomitant with the insertion of a domain of SecA into the membrane (Figure 7, step 1). This insertional mechanism for SecA was proposed based on the finding that a 30 kDa C-terminal domain of SecA becomes protease-resistant when SecA binds ATP and preprotein (Economou and Wickner, 1994; Economou *et al.*, 1995). Hydrolysis of ATP subsequently relieves the protease resistance of the SecA domain, and this process has been attributed to the de-insertion of the SecA domain (Economou and Wickner, 1994) (Figure 7, step 2) while it releases the bound precursor protein. At that stage, SecA can re-bind to the exposed portion of the partially translocated preprotein, and this step allows the translocation of another 2.5 kDa polypeptide segment (Figure 7, step 3). Therefore, translocation will only be partly driven by the SecA co-insertion reaction while the remainder results from the preprotein binding reaction and an associated conformational change of SecA.

Particularly interesting is the impact of azide on the catalytic cycle of the translocase. Azide inhibits translocation by blocking the translocation ATPase activity of SecA (Oliver *et al.*, 1990). It does not interfere with binding of nucleotides at NBS-I, as it did not affect the photocrosslinking of [α-^{32}P]ATP to NBS-I and the formation of the staphylococcal protease V8-resistant conformation of SecA in the presence of a NBS-I saturating concentration of ATP or ATPγS (J.van der Wolk, unpublished data). NBS-I has been implicated in the ATP-dependent membrane insertion/de-insertion of SecA (Economou *et al.*, 1995). Consistent with these findings is the observation that azide does not diminish the ATP- and preprotein-dependent formation of the proteinase-resistant 30 kDa SecA fragment. Rather, it promotes the formation of the 30 kDa SecA fragment, indicating that azide stabilizes the membrane-inserted state of SecA. On the other hand,

azide blocks the de-insertion reaction as monitored by the ATP-dependent chase of membrane-inserted ^{125}I-labelled SecA by an excess of unlabelled SecA. This step requires hydrolysis of ATP at NBS-I (Economou *et al.*, 1995) and, therefore, it seems that azide inhibits this reaction specifically. Strikingly, in the presence of azide, a number of new intermediates are observed that are uniquely positioned in between the stable intermediates. In the presence of azide, intermediates appear to be spaced at intervals of ~2.5 kDa instead of 5 kDa. Since azide traps the SecA in a transitional state during the translocation reaction, these new intermediates may resemble such transitional states as well. This is expected, for instance, when the ATP-dependent membrane insertion of SecA is a rate-determining step in translocation. In the absence of azide, intermediates will accumulate at the step that precedes the SecA membrane insertion, and thus mainly result from the translocation progress effected by SecA preprotein binding. However, in the presence of azide, ATP hydrolysis is blocked and SecA is retained in the membrane-inserted state for longer times, yielding intermediates associated with this state. The distribution, i.e. the intensity of the various intermediates, is determined by the relative rates of SecA membrane insertion, de-insertion and preprotein release, and the preprotein (re-)binding reaction. The observation that azide reduced the 'apparent' step size 2-fold is in agreement with a two-step translocation reaction as discussed above.

Recently, it was suggested that the stepwise translocation of proOmpA is caused solely by the short hydrophobic segments present in proOmpA (Sato *et al.*, 1997). Due to the association with the translocase, such hydrophobic segments would retard translocation and give rise to temporarily arrested or delayed states. However, stepwise translocation of proOmpA derivatives devoid of hydrophobic segments also occurred and, albeit with lower efficiency, the intermediates I_{16}, I_{21}, I_{26} and the artificially constructed I_{29} were prominently present. The phenomenological step size of translocation is probably not fixed but irregular, due, for example, to hysteresis movements of the partially translocated polypeptide chain (Schiebel *et al.*, 1991; Driessen, 1992). Such movements may be brought about by differences in the hydrophobicity of the translocating segments (Sato *et al.*, 1997) and/or the folding of translocated domains (Arkowitz *et al.*, 1993). The mechanistic step size, however, must be a fixed value as this is determined by the cognitive characteristics of the ATP-dependent molecular motor that drives translocation. In this respect, SDS–PAGE analysis only provides an estimate of the step size.

The mechanism by which Δp stimulates translocation has remained obscure as it appears to affect multiple stages of the translocation reaction (Driessen, 1994). Δp can drive the efficient translocation of large polypeptide domains in the absence of SecA association (Schiebel *et al.*, 1991), suggesting that the step size may be discontinuous when both energy sources are present. However, this efficient Δp-driven translocation reaction has been observed under artificial conditions, i.e. when SecA is removed from the translocation sites. In the presence of SecA, ATP and a Δp, translocation is extremely fast and seems to occur without the appearance of intermediates. However, it remains to be established if the ATP-driven translocation of proOmpA in the presence of a Δp is indeed a continuous process, rather than being stepwise as observed for SecA-mediated translocation alone. In contrast to native *E.coli* D10 IMVs, the stimulatory effect of a Δp with KM9 IMVs is only pronounced when either SecA or ATP is present at limiting concentrations. Dissipation of the Δp with CCCP or the ionophores valinomycin and nigericin blocks translocation at a very early stage in D10 IMVs, supporting the notion that the late stages of proOmpA translocation are indeed Δp-dependent (Schiebel *et al.*, 1991). Again, this phenomenon is less evident with KM9 IMVs. The exact reason for this discrepancy is not clear. In this respect, the cold-sensitive phenotype of Δ*secG* null mutants has been reported to be strain dependent (Bost and Belin, 1995) and, in some genetic backgrounds, the growth defect of this mutation is only manifested when the *unc* genes coding for the F_1F_0-ATPase are also deleted (Duong and Wickner, 1997a). Moreover, SecG appears to be critical for translocation in the absence of a Δp (Nishiyama *et al.*, 1994, 1996). Our observations of the difference in Δp dependency of translocation into KM9 and D10 IMVs may be related to the function of SecG.

In conclusion, SecA-driven preprotein translocation is a stepwise process. Each turnover of the translocase, which involves binding and hydrolysis of ATP, results in the translocation of ~40–50 amino acid residues of the preprotein across the membrane. This process is driven by two distinct and consecutive enzymatic steps utilizing respectively the energies of preprotein and ATP binding to SecA.

Materials and methods

Bacterial strains and growth conditions

Unless indicated otherwise, *E.coli* strains D10 (*rna*10, *rel*A1, *spo*T1, *met*B1) and KM9 (*unc*$^-$::Tn*10*, *rel*A1, *spo*T1, *met*B1) (Klionsky *et al.*, 1984) were grown at 37°C in Luria Bertani (LB) broth supplemented with 100 μg/ml of ampicillin, 0.5% (w/v) glucose, or 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), as required. Cloning and plasmid constructions were done in *E.coli* JM101 or JM110 (Yanish-Perron *et al.*, 1985). Uracil-containing single-stranded template DNA for oligo-directed mutagenesis was obtained by growing the corresponding M13KO7 helper phage on *E.coli* CJ236 (Raleigh *et al.*, 1988). Overexpression of the SecYEG complex and leader peptidase (LepA) was in *E.coli* strain D10 harbouring plasmid pET324 (Van der Does *et al.*, 1996) or pTD101 (Date and Wickner, 1981), respectively.

Biochemicals

SecA (Cabelli *et al.*, 1988), SecB (Weiss *et al.*, 1988), proOmpA (Crooke *et al.*, 1988) and SecYEG (Brundage *et al.*, 1990) were purified as described. [^{35}S]proOmpA was synthesized from plasmid pET033 using an *in vitro* transcription–translation reaction (De Vrije *et al.*, 1987), and affinity-purified as described (Crooke and Wickner, 1987). SecA was iodinated with Na^{125}I as described (Economou and Wickner, 1994; Den Blaauwen *et al.*, 1997). Inverted IMVs were prepared from *E.coli* strain D10 and KM9 by the procedure of Chang *et al.* (1978) and, when indicated, treated with 6 M urea (Cunningham *et al.*, 1989).

DNA manipulation and oligonucleotide-directed mutagenesis

The procedure used for site-directed mutagenesis of the *ompA* gene was essentially as described by Kunkel *et al.* (1987). Mismatch oligonucleotides, resulting amino acid substitutions and the introduction of restriction endonuclease digestion sites are shown in Table I. The *Eco*RI–*Pst*I restriction fragment of pRD87 harbouring the *ompA* gene was cloned in pUC18 to yield pET149. This plasmid was used to express wild-type proOmpA. Mutagenesis was done on single-stranded DNA containing the *Hin*cII–*Bam*HI and *Bam*HI–*Pst*I restriction fragments of pET149,

Table I. Mismatch primers used for oligonucleotide-directed mutagenesis

Primer	Sequence[a]	Amino acid substitution
OMW13	5′-AGTGGCACTG**ATG**GGTTTCGCTACCGTAGCGC-3′	$Ala^{13}Met$
OMW121	5′-TGCACGCCATACG**GCG**CCACCCAGACG-3′	$Met^{121}Ala$
OMW182	5′-ACCCAGGCTCAG**GCG**CCGTTGTCCGG-3′	$Met^{182}Ala$
OMW300	5′-GTTGGATTC**GCC**GGCTCCTCTTGCGGAGATCTTG-3′	$Met^{300}Ala$

[a]Nucleotides in bold and underlined mark the mismatches in the DNA sequence of the oligonucleotides and the introduced *Nar*I endonuclease restriction sites, respectively.

which were cloned in the pBluescript KS(+)/SK(+) series (Stratagene Cloning Systems, La Jolla, CA). Mutagenized fragments were reintroduced into pET149, from which the corresponding *Hin*cII–*Pst*I fragment was removed, to yield pET023 (Figure 1) in which Met121, Met182 and Met300 have been replaced by Ala.

Replacement of Ala13 in proOmpA by Met was done by two-step PCR as described by Landt *et al.* (1990). A 0.3 kb *Hin*cII–*Eco*RV restriction fragment from pRD87 was cloned into the *Eco*RV site of pBluescript SK(+). The resulting plasmid pET133 was used as a template to amplify a 171 bp DNA fragment corresponding to the 5′-end of the *ompA* gene using the oligonucleotide forKS (5′-TGGGTACCG-GGCCCCCCC-3′) as 5′-primer and OMW13 (Table I) as mismatch primer. The resulting DNA fragment was isolated and, together with revKS (5′-GAACTAGTGGATCCCCCG-3′), used in a second PCR as primer, in which pET133 again was used as a template. From the resulting 384 bp PCR product, a 225 bp *Hin*cII–*Pst*I restriction fragment was isolated, and ligated into pET023, from which the corresponding *Hin*cII–*Pst*I fragment was removed. The resulting plasmid pET033 was used to express the modified proOmpAM3 protein (Figure 1). All mutagenized DNA fragments were sequenced on a Vistra DNA sequencer 725 (Amersham, Buckinghamshire, UK).

In vitro translocation of ProOmpA

In vitro translocation of [^{35}S]proOmpA (in 50 µl) was performed at 37°C as described (Cunningham *et al.*, 1989) with 20 µg/ml of SecA, 32 µg/ml of SecB, 1 µl of urea-denatured [^{35}S]proOmpA, 10 mM phosphocreatine and 50 µg/ml creatine kinase in buffer B [50 mM HEPES KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml bovine serum albumin (BSA), 10 mM dithiothreitol and 2 mM $Mg(OAc)_2$]. *Escherichia coli* D10 or KM9 inverted IMVs were added to a final concentration of 300 µg/ml and, as indicated, the reactions were initiated by the addition of 2 µM or 2 mM ATP. At various time points, translocation was terminated by chilling on ice. Samples were treated with proteinase K (0.1 mg/ml) for 15 min on ice, precipitated with 7.5% (w/v) trichloroacetic acid, washed with ice-cold acetone and solubilized in SDS sample buffer. Samples were analysed by 16% High Tricine SDS–PAGE (Schägger and Von Jagow, 1987) to separate polypeptide fragments in the range of 3–30 kDa or, when indicated, by conventional 12% SDS–PAGE. Gels were dried and exposed to Kodak Biomax MR film. Autoradiograms were scanned densitometrically using a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and analysed using SigmaScan/Image (Jandel Corp., San Rafael, CA).

In separate translocation reactions in which the [^{35}S]proOmpA was replaced by unlabelled proOmpA, the concentrations of ATP and ADP were determined by including a trace amount of [α-^{32}P]ATP. Radiolabelled nucleotides were separated on thin-layer chromatography using Polygram gel300 PEI/UV254 plates (Machery-Nagel, Düren, Germany) and 0.65 M KH_2PO_4 as eluents. After air-drying, spots were quantified by the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Other techniques

Protein determination was performed according to Lowry *et al.* (1951) with BSA as standard. Generation of a $\Delta\psi$ (inside positive) and ΔpH (inside acid) was followed with the fluorescent indicators bis(3-propyl-5-oxoisooxazol-4-yl)pentamethine oxonol (Oxonol VI) (Ex/Em, 599 nm/634 nm) (Apell and Bersch, 1987) and 9-amino-6-chloro-2-methoxyacridine (ACMA) (Ex/Em, 409 nm/474 nm) (Klionsky *et al.*, 1984). The nucleotide occupancy of NBS-I and -II was calculated as described by Den Blaauwen *et al.* (1996) using K_d values of 0.15 and 340 µM, respectively. The insertion and de-insertion of SecA were monitored by following the formation of the 30 kDa trypsin-protected fragment of ^{125}I-labelled SecA (Economou and Wickner, 1994; Den Blaauwen *et al.*, 1997).

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